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Molecular Sex Determination of Captive Komodo Dragons (*Varanus komodoensis*) at Gembira Loka Zoo, Surabaya Zoo, and Ragunan Zoo, Indonesia

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Captive breeding of endangered species is often difficult, and may be hampered by many factors. Sexual monomorphism, in which males and females are not easily distinguishable, is one such factor and is a common problem in captive breeding of many avian and reptile species. Species-specific nuclear DNA markers, recently developed to identify portions of sex chromosomes, were employed in this study for sex determination of Komodo dragons (*Varanus Komodoensis*). Each animal was uniquely tagged using a passive integrated micro-transponder (TROVAN 100A type transponders of 13 mm in length and 2 mm in diameter). The sex of a total of 81 individual Komodo dragons (44 samples from Ragunan zoo, 26 samples from Surabaya zoo, and 11 samples from Gembira Loka zoo) were determined using primers Ksex 1for and Ksex 3rev. A series of preliminary PCR amplifications were conducted using DNA from individuals of known sex. During these preliminary tests, researchers varied the annealing temperatures, number of cycles, and concentrations of reagents, in order to identify the best protocol for sex determination using our sample set. We thus developed our own PCR protocol for this study, which resulted in the amplification of band A in females and band C in males. Results from band B, however, turned out to be non-determinative in our study because, for females, band B was not always visible, and for males sometimes a similar, but lighter band was also amplified, making interpretation difficult. In this study, sex determination was based mainly on the difference in size between the female-specific 812 bp fragment and the homologous, longer fragment amplified for males.

Keywords: captive breeding, zoo, komodo dragon, molecular sexing, PCR

INTRODUCTION

Sex identification provides valuable insights into species breeding strategies, which informs conservation and management programs (Helander *et al*. 2007; Garcia *et al*. 2009; Naim *et al*. 2011) and reproduction programs for threatened species (Ellegren & Sheldon 1997). The most basic and important information in establishing captive breeding programs is a clear understanding of sex ratio; that is, the number of males and females in a population. Sex determination in captivity allows individual identification for mating, and it is useful in order to set breeding procedures on the basis of data on male-to-female ratios available from field studies. However, sex determination is often hampered by the occurrence of sexual monomorphism, in which the male and female are not easily distinguished. Komodo dragons have long been popular zoo attractions, thanks to their size and fearsome reputation. The Komodo dragon *Varanus komodoensis* is the world's largest lizard, up to 3 m in length and over 70 kg in weight. It is restricted to five small islands in Eastern Indonesia (Ciofi & De Boer 2004). It is endemic to southeast Indonesia and protected throughout its range, which includes Komodo National Park and the island of Flores. The species is now listed in CITES Appendix 1, and has been proposed for designation as "endangered" on the IUCN red list (Ciofi & De Boer 2004). Although males tend to grow bulkier and bigger than females, Komodo dragons have no obvious morphological differences between sexes except in the arrangement of a specific part of precloacal scales. So far, however, examination of the precloacal scales to determine sex has been proved to be difficult in the field. The scale pattern is not always clear, and probing the cloaca for presence or absence of inverted hemipenes is troublesome since females have hemiclitoreal sacs at approximately the same position of males hemipenes, and gender can often be confused. Sexing Komodos remains a challenge to researchers; the dragons themselves appear to have little trouble figuring out who is who.

It is relatively easy to restrain Komodo dragon in captivity, so a number of accurate alternative tests can be used to determine sex. Laparoscopy provides direct visualization of gonads by means

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of an endoscope. Radiography of the cloacal region shows the presence or absence of the bones in males associated with the hemipenis. Differences in testosterone concentrations can be assessed in fresh blood samples. Finally, ultrasound can be used to assess whether ovarian follicles are present. Although accurate, these techniques have not given consistent results for young animals of under 10 months of age, and their employment is sometimes hampered by the requirement for quality equipment. It is therefore important to have an accurate and relatively non-expensive technique available for sex determination. Thus far, DNA technology has provided the most consistent results (Halverson & Spelman 2002).

Animal molecular sexing techniques are usually based on polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) sequences from the Y (mammal) or W (bird) chromosome-linked loci (Putze *et al*. 2007), with concomitant amplification of X or Z, respectively. Alternatively, sexing techniques are based on differences in the PCR yielded fragment of the X-Y homologous gene amelogenin from the X and Y chromosome. Nevertheless, X and Z chromosome-linked loci are not usually used as stand-alone techniques for animal sex determination (Dubiec & Zagalska-Neubauer 2006). Sexing technique methods are usually based either on the presence or absence of PCR amplification products, on the differences in band lengths and/or the resulting banding patterns on gel, and/or on differences in sequences ('qualitative sexing'), which enable researchers to distinguish between X and Y chromosomes (mammals) or W and Z chromosomes (birds) [restriction fragment length polymorphisms (RFLPs, Sacchi *et al*. 2004), amplified fragment length polymorphism (AFLPs, Griffiths & Orr 1999), single strand conformation polymorphism (SSCPs, Ramos *et al*. 2009), microsatellite alleles (Nesje & Roed 2000), and oligonucleotide-microarrays (Kalz *et al*. 2006; Wang *et al*. 2008)].

Sex determination tests using DNA markers have been used in many vertebrate species, including whales (Baker *et al*. 1991), birds and reptiles (Millar *et al*. 1996, 1997; Fleming *et al*. 1996). Reptiles exhibit different modalities of gender determination, including both temperature-dependent determination and chromosomal sex determination. In regards to chromosomal determination, some genera have XY sex chromosomes, some genera have ZW sex chromosomes (such as in Varanus; Olmo 1986), and some genera have no recognizable sex chromosomes. Indeed, some reptile families show all three variations. It is unlikely that a DNA marker found in one genus will be sufficiently conserved that it can be broadly applied in other reptiles. Within a single genus, however, sometimes markers for sexing one species can be applied to other species. However, until recently, no sex determination markers where available for the genus *Varanus*. For Komodo dragons, species-specific DNA markers for sex determination have been developed by Halverson and Spelmann (2002) to identify portions of the heteromorphic female sex chromosomes. These were the first probes for sex determination in varanid lizards.

The goal of this study was to determine the sex of captive Komodo dragons kept at the Zoos in Surabaya (Surabaya zoo), Yogyakarta (Gembira loka zoo), and Jakarta (Ragunan zoo). The sex of individual Komodo dragons in captivity was identified using a pair of PCR primers (Ksex 1for and Ksex 3rev) as decribed by Halverson and Spelmann (2002).

MATERIALS AND METHODS

Animal Marking. A total of 81 Komodo dragons were studied, including 44 Komodo dragons kept in Ragunan zoo (Jakarta), 26 Komodo dragons kept in Surabaya zoo (Surabaya), and 11 Komodo dragons kept in Gembira loka zoo (Yogyakarta). The zoos of Ragunan, Gembira loka, and Surabaya are the three largest Zoos in Indonesia and hold the most easily accessible populations of Komodo dragons.

Each animal was uniquely tagged using a passive integrated micro-transponder. We used TROVAN 100A type transponders of 13 mm in length and 2 mm in diameter. Each transponder easily scanned by an appropriate reader-contains a 10 digit barcode that is uniquely assigned to the tagged animal. Transponders were inserted under the skin of the upper part of the right hind leg of the animal (Figure 1). Most animals, however, already had transponders in the upper portion of one of the front legs, implanted previously by zoo staff. Therefore, animals were checked for the presence of signal from an existing transponder and, if no signal was detected, a new device was inserted in the upper part of the right hind leg of the animal. This technique provides permanent tagging with no injury to the animal being treated.

Measurement of Snout-Vent Length (SVL). After tagging, Snout-Vent Length (SVL, cm) was measured for each animal. This is a standard measurement of body length taken from the tip of the nose (snout) to the anus (vent), excluding the tail

(http://www.sdnhm.org/fieldguide/herps/glossary.html). Laver *et al*. (2012) used SVL-defined as the straight line distance measured between the tip of the snout and the cloaca to the nearest millimeter-as the measure of growth for each individual. In our study, measurements were made using a flexible fiberglass tailor's tape.

Sampling Procedure. Blood was collected from the caudal vein, using a 25Gx⅝" or a 23Gx1" disposable needle, depending on the size of the specimen. The needle was inserted laterally, at about 10-30 cm, depending on the size of the animal (Figure 2). Approximately 100 microliters (µL) of blood were collected and preserved at ambient temperature in 2 milliliter (mL) tubes containing a lysis buffer made of 0.1 M Tris buffer, 0.1 M EDTA, 0.2 M NaCl and 1% sodium dodecyl sulfate, pH 8.0. Blood samples were transported to the genetic laboratory of Zoology at the LIPI Research Center for Biology, and stored at -20 ºC.

DNA Extraction and DNA Quality Assessment. DNA was extracted from whole blood samples using standard phenol/chloroform procedures (Sambrook *et al*. 2001) based on disruption of red and white cell membranes, removal of protein contaminants with organic solvents, and isolation of high molecular weight nucleic acids in the presence of salt and ethanol. A Phase Lock Gel™ light (Eppendorf) was used for a more efficient separation of organic and aqueous phases. DNA was stored at -20 ºC and then deposited at the DNA Bank for Indonesian Fauna, in the genetic laboratory of Zoology at the LIPI Research Center for Biology.

DNA quality was assessed by visualizing nucleic acids in agarose gel under ultraviolet light following electrophoretic reaction. Electrophoresis is a process by which nucleic acids move along an electric field. The DNA are run through an agarose gel, a complex network of polymeric molecules whose average pore size depends on the buffer composition and the type and concentration of agarose used. The gel is placed in the electrophoretic apparatus, which consists mainly of a buffer reservoir in the form a square tank with a positive and negative electrodes placed at the opposite sites of the tank. Agarose gels are usually run in an electric field of constant strength and direction under 100 V of power. At neutral pH values, the DNA is negatively charged so that fragments loaded into a sample well at the cathode end of a gel move through the gel towards the anode end.

Figure 1. From left to right: 13 mm long passive integrated transpoder (PIT), insertion of PIT, checking for PIT presence with dedicated reader.

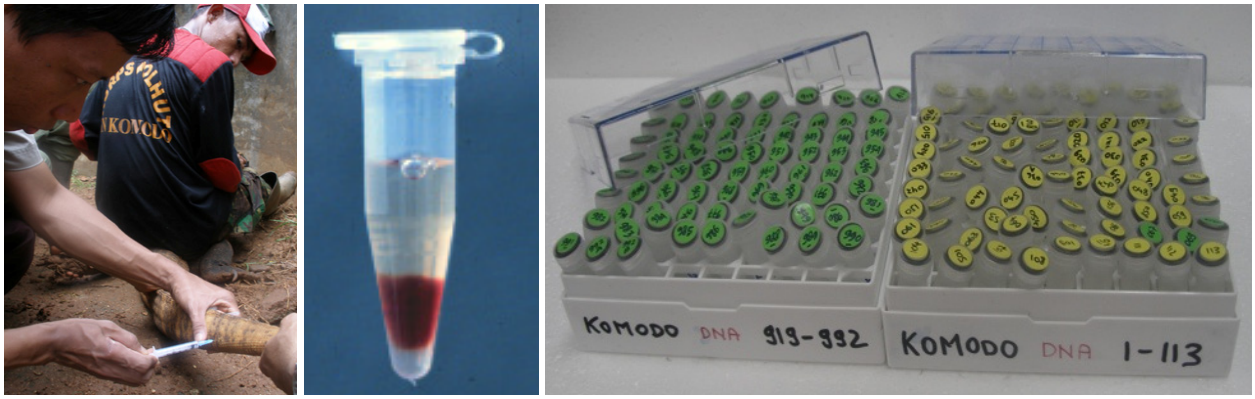

Figure 2. From left to right: Collection of blood sample; blood sample (approximately 100 uL) in 1.5 mL collection tube, and DNA sample.

PCR Amplification of Sex-Specific DNA Fragments. Species-specific nuclear DNA markers, recently developed by Halverson and Spelman (2002) were employed for sex determination of 81 Komodo dragons in captivity. In PCR, oligonucleotide probes, or primers, anneal to a template of DNA from a single individual. A polymerase that does not denature at high temperatures, called Taq polymerase, catalyzes the replication of that DNA. Changing the temperature controls how the reaction proceeds: the primers anneal, the DNA is replicated, and then the two strands are denatured to make them available for more replication. Through a repeated cycling of temperatures, the template is amplified to make millions of copies. Female Komodo dragons are heterogametic, with one Z chromosome and one W chromosome (Olmo 1986). DNA samples are therefore characterized by alleles of different size, detected in the gel as two distinct bands. Males are homogametic (they have two Z chromosomes) and have alleles of the same size, so that PCR products will run at the same speed during electrophoresis. The two alleles will overlap each other in the gel, and will be therefore represented by a single band. This is the result of PCR primers annealing to both sex chromosomes but amplifying DNA fragments of different lengths.

One set of forward (Ksex 1for) and reverse PCR primers (Ksex3rev) were used (Figure 3) and PCR conditions were optimized for annealing temperature, magnesium concentration, and polymerase. In this study, PCR amplification produced a fragment of 812 base pairs (bp) for females, in accordance with the length of the cloned sequence (Figure 4). The polymerase chain reaction (PCR) protocol for amplification of Komodo dragon sex-related DNA sequences featured the following conditions: 10 µL total volume reaction containing 1x reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 25 µM of each primer and 0.05 U of Taq DNA polymerase (Fermentas). PCR cycling was performed in the Applied Biosystems GeneAmp PCR system 2700 thermocycler for an initial denaturation step at 95 ºC for 5 min, followed by 35 cycles of 30 s at 95 ºC, annealing for 30 s at 57 ºC and extension for 1 min at 72 ºC, with a final extension step of 10 min at 72 ºC.

Visualisation of PCR Product for Sex Determination. PCR products were separated by electrophoresis at 100 V for 30 min using a 2% agarose gel. Because DNA is negatively charged, electrophoresis causes PCR products to move through the matrix of an agarose gel towards a positive electrode. The shorter strands move faster, so when there are several DNA sequences of different lengths amplified during PCR, electrophoresis separates PCR products. The Agarose gel is left staining in a buffer solution containing ethidium bromide, a dye that intercalates in DNA and makes the separated PCR products visible under UV light. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. The PCR

```
      Ksex 1for →
Tgtccatgccacgcgggcaaaagcggctcatgactgcagatgtaggggtcagaatgacttaaccttgt
Ttgctttgagccaaaaggggagattgggcttggctcgcgaccaaaatcggctcgcgaccacgtttgta
Aaggtcagaatggattaagtttatgctcatcgaaacttatggagaaatagggcttggctcgcgggaaa
Aatcggatcgtgaccacttttgcagttgtgagactggattgacctcattcgcatcgaacgttatgggg
Aaattgtcatgcctcccgaccaaaatcggctcgcgatcacgtttgtaaaggtcagaatgcatttacct
Aatttgcaacgtaccttatgtggaaattggacttatctctcgacaaagataggtttacgaagacgttt
Gcggcggtcagaatggaatgacctaatttccattgaaccttatcaggaaattatcatgcatcgcgacc
Taaatcagctagcgaccacatttttcggggtcataatggatcaagtatattcacatcgaacctgatgg
Ggaaattggacttggctcatgaccaaaatcagcttgcgagcgtgtctgtagtggtcagaaaggattga
Ccttctttctatcgaaccttatggggaaatgttcttgcctcgtgggcaaaatcggctctcgaccacat
Ttgtgaaggtcggaatgaattagccttatttgcatataacctaatggggaaattggacctgcctcgct
acaaaaataggctcgtgacgaggtttgtaggggtcagaatgaattaagtttattggcatggaca
                                                    ← Ksex 3rev
```

Figure 3. Sequence from the W chromosome of *Varanus komodoensis*. Ksex1for (forward) and Ksex3rev (reverse) PCR primer sequences are underlined and highlighted in grey.

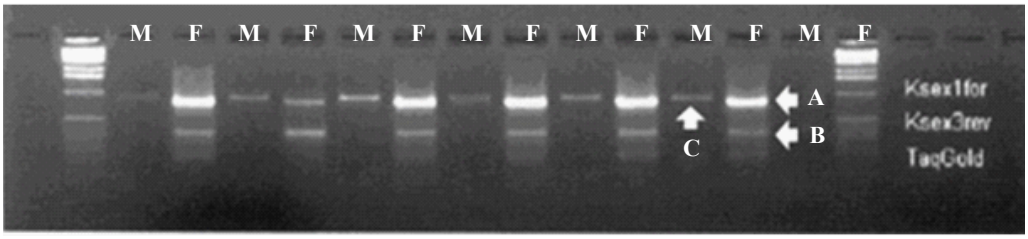

Figure 4. Agarose electrophoresis of PCR amplification products of known sex Komodo Dragon DNA with Ksex 1for and Ksex 3rev. (M: males, F: females).

products appear as bands along the column where they migrated.

The visualization product comparing the male and female pools is shown in Figure 4. Female is indicated by presence of band A. Band B is also restricted to females and, while not predictive of sex, it probably arises from a less homologous binding of primer Ksex 1for. In males, only one band, less intense and about 100 bp longer than female-specific bands, is produced (band C in Figure 4). Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. The longer sequence amplified in males may represent a longer, but similar sequence in the male Z chromosome with a similar annealing sites for Ksex 1for and Ksex 3rev. Alternatively, a mutation event in the male homologous Z chromosome sequence may have resulted in the optimal annealing site for either of the two primers to be located about 100 bp outside the original target sequence.

RESULTS

Snout-Vent Length (SVL). Measurement of Snout-Vent Length (SVL) is presented in Tables 1,

Table 1. Summary of sex determination results for Komodo dragons at Ragunan zoo. Animal identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

ID	Cage	Date	PIT	Arrival	Date of birth	Life stage	SVL	Sex
KR1	Surplus	28-Mar-06	00-01F6-63FD		19-Feb-98	F1	87.00	Female
KR2	Surplus	28-Mar-06	00-01F7-D7B4		10-Mar-98	F1	87.50	Female
KR3	Surplus	28-Mar-06	00-0648-49AB	5-May-96		F0	125.00	Male
KR4	Penangkaran	28-Mar-06	00-0142-0DDB		19-Feb-98	F1	97.70	Female
KR5	Penangkaran	28-Mar-06	00-01F7-872D		15-Apr-98	F1	101.00	Female
KR6	Penangkaran	28-Mar-06	00-01E5-C3BB		19-Feb-98	F1	95.00	Male
KR7	Penangkaran	28-Mar-06	00-01F7-D567		19-Feb-98	F1	117.00	Male
KR8	Penangkaran	28-Mar-06	00-01E5-BFEA		19-Feb-98	F1	100.00	Female
KR9	Penangkaran	28-Mar-06	00-01F7-8573		19-Feb-98	F1	102.50	Female
KR10	Penangkaran	28-Mar-06	00-01F7-8468		19-Feb-98	F1	118.00	Male
KR11	Penangkaran	29-Mar-06	00-01F6-60E9		19-Feb-98	F1	85.00	Female
KR12	Penangkaran	29-Mar-06	00-0128-935E		19-Feb-98	F1	101.00	Female
KR13	Penangkaran	29-Mar-06	00-0128-B47A		19-Feb-98	F1	96.00	Female
KR14	Penangkaran	29-Mar-06	00-01E5-701A		19-Feb-98	F1	96.00	Female
KR15	Penangkaran	29-Mar-06	00-0128-9AFA		19-Feb-98	F1	98.00	Female
KR16	Penangkaran	29-Mar-06	00-0127-BC77		10-Mar-98	F1	90.00	Female
KR17	Penangkaran	29-Mar-06	00-0127-BA1C		19-Feb-98	F1	95.00	Female
KR18	Penangkaran	29-Mar-06	00-01F7-83D1		19-Feb-98	F1	105.00	Female
KR19	Penangkaran	29-Mar-06	00-01F7-7E59		19-Feb-98	F1	104.50	Female
KR20	Penangkaran	29-Mar-06	00-01F6-3030		15-Mar-98	F1	117.00	Male
KR21	Untung 2	29-Mar-06	00-01F6-55FF		10-Mar-98	F1	85.00	Female
KR22	Suhada 1	29-Mar-06	00-01E5-7771		10-Mar-98	F1	113.00	Male
KR23	Suhada 1	29-Mar-06	00-01E5-808A		10-Mar-98	F1	82.00	Female
KR24	Suhada 2	29-Mar-06	00-01E5-7FA9		10-Mar-98	F1	87.50	Female
KR25	Suhada 2	29-Mar-06	00-0128-B7A5		10-Mar-98	F1	91.00	Female
KR26	Suhada 2	29-Mar-06	00-0128-9380		10-Mar-98	F1	101.00	Male
KR27	Suhada 2	29-Mar-06	00-0127-C179		10-Mar-98	F1	105.00	Male
KR28	Suhada 2	29-Mar-06	00-01F7-8852		10-Mar-98	F1	96.50	Female
KR29	Suhada 3	29-Mar-06	00-0127-C315		10-Mar-98	F1	96.00	Female
KR30	Suhada 3	29-Mar-06	00-0128-B4B7		10-Mar-98	F2	92.00	Female
KR31	Suhada 3	29-Mar-06	00-0648-3B4B	8-May-96		F0	105.00	Male
KR32	Suhada 3	29-Mar-06	00-01F7-C992		10-Mar-98	F1	104.00	Male
KR33	Suhada 4	29-Mar-06	00-0127-D5E5		22-Jun-89	F1	111.00	Male
KR34	Suhada 5	29-Mar-06	00-066F-EA3F		22-Jun-89	F1	144.00	Male
KR35	Suhada 4	29-Mar-06	00-0128-B7D2	9-May-96		F0	118.00	Female
KR36	Untung 2	29-Mar-06	00-0142-0D7D		15-Apr-98	F1	117.00	Male
KR37	Untung 2	29-Mar-06	00-01E5-80EA		15-Apr-98	F1	97.50	Female
KR38	Untung 1	29-Mar-06	00-012B-667C	8-May-96		F0	126.00	Female
KR39	Untung 1	29-Mar-06	00-066C-8E3D	8-May-96		F0	140.00	Male
KR40	Untung 1	29-Mar-06	00-0670-00D2	8-May-96		F0	117.00	Female
KR41	Untung 1	29-Mar-06	00-0128-9F28	9-May-96		F0	125.00	Female
KR42	Untung 3	29-Mar-06	00-0127-C5A5	9-May-96		F0	121.00	Female
KR43	Nursery	29-Mar-06	00-066C-F2FB		6-Jun-05	F2	30.00	Female
KR44	Untung 3	03-Apr-06	00-0127-E61F	9-May-96		F0	105.00	Female

2, and 3. The average size was 115.1 cm for males and 97.2 cm for females in Ragunan zoo; 101.4 cm for males and 79.1 cm for females in Surabaya zoo; 91 cm for males and 86 cm for females in Gembira loka zoo.

Sex of Komodo Dragon in Zoo Captivity. A series of preliminary PCR amplifications were conducted on some samples of known sex, to test for the best protocol for all our DNA samples of Komodo dragons. An example of these results is reported in Figure 5. For males, PCR always resulted in the amplification of the male-specific longer fragment and would sometimes produce the 812 bp fragment specific for females. The first one corresponded to the original annealing site described for the female-specific sequence and the second one was located about 100 bp outside the original target sequence. The first annealing site appears to be not fully complementary to the primer sequence because it does not always result in a successful amplification and, when it does, it often provides PCR products of lighter intensity.

The pattern of sex identification showed in Figure 5 was used subsequently to determine sex of 81 Komodo dragons from Ragunan zoo, Surabaya zoo, and Gembira loka zoo. The sex of

Table 2. Summary of sex determination results for Komodo dragons at Surabaya zoo. Identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

ID	Cage	Date	PIT	Arrival	Date of birth	Life stage	SVL	Sex
KS1	Depan parker	12-Apr-06	00-01D3-D079		27-Apr-02	F2	86.00	Male
KS2	Depan parker	12-Apr-06	00-01D2-BEFF		1-Apr-03	F2	67.00	Female
KS3	Depan parker	12-Apr-06	00-01C4-914A		4-Apr-03	F2	68.00	Male
KS4	Depan parker	12-Apr-06	00-012A-01FE		3-Apr-03	F2	72.00	Female
KS5	Depan parker	12-Apr-06	00-0143-616B		7-Apr-03	F2	67.00	Female
KS6	Depan parker	12-Apr-06	00-012E-9FBC		7-Apr-03	F2	64.00	Female
KS7	Depan parker	12-Apr-06	00-0143-70BD		2-Apr-03	F2	59.00	Female
KS8	Depan parker	12-Apr-06	00-01D3-1AF0		3-Apr-03	F2	60.00	Female
KS9	Depan parker	12-Apr-06	00-0125-1983		25-Jun-02	F2	83.00	Male
KS10	Depan parker	12-Apr-06	00-0135-0BCD		8-Apr-03	F2	64.00	Female
KS11	Depan parker	12-Apr-06	00-0143-6514		14-Apr-03	F2	72.00	Female
KS12	Depan parker	12-Apr-06	00-01D2-BE63		12-Apr-03	F2	62.00	Female
KS13	Depan parker	12-Apr-06	00-013C-4FA9		25-Jun-02	F2	79.00	Male
KS14	Depan parker	12-Apr-06	00-0143-4886		15-Apr-03	F2	66.00	Female
KS15	Depan parker	12-Apr-06	00-01D2-BCD8		29-Apr-02	F2	83.00	Male
KS16	D-Onta T	12-Apr-06	00-01D3-D8FF			F1	121.00	Female
KS17	Depan WC	12-Apr-06	00-01D2-BE18	16-Jun-92		F1	113.00	Female
KS18	Depan WC	12-Apr-06	00-0135-106B	~1999		F0	123.00	Male
KS19	Depan Zebra	12-Apr-06	00-01C4-973D		17-Jun-97	F1	99.00	Female
KS20	Depan Zebra	12-Apr-06	00-012A-0973	~1999		F0	128.00	Male
KS21	Depan Zebra	12-Apr-06	00-01D2-BE74		28-Apr-98	F1	125.00	Male
KS22	Depan Zebra	12-Apr-06	00-0143-5BB9	~1999		F0	120.00	Male
KS23	Depan Zebra	12-Apr-06	00-01D3-D02D	~1999		F0	119.00	Male
KS24	Depan Zebra	12-Apr-06	00-0143-7122			F1	94.00	Female
KS25	Depan Zebra	12-Apr-06	00-01C4-98E4	1-Apr-96	19-Jul-95	F1	89.00	Female
KS26	Depan Zebra	12-Apr-06	00-013C-4F3E	~1999		F0	97.00	Female

Table 3. Summary of sex determination results for Komodo dragons at Gembira Loka zoo. Identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

ID	Cage	Date	PIT	Arrival	Date of birth	Life stage	SVL	Sex
KY1	Karantina	3-May-06	116233544A			F2	86.00	Male
KY2	Karantina	3-May-06	116244117A			F2	76.00	Male
KY3	Karantina	3-May-06	114672246A			F2	60.00	Male
KY4	Kandang 4	3-May-06	115752652A			F2	87.00	Male
KY5	Kandang 4	3-May-06	116152164A			F2	70.00	Male
KY6	Kandang 4	3-May-06	116312321A			F2	72.00	Female
KY7	Kandang 4	3-May-06	116335752A			F2	76.00	Male
KY8	Kandang 5	3-May-06	114673364A			F1	119.00	Male
KY9	Kandang 5	3-May-06	114655792A			F1	100.00	Female
KY10		3-May-06	00-0634-AA5E			F0	132.00	Male
KY11		3-May-06	00-0648-4858			F0	113.00	Male

all 81 individual Komodo dragons were determined successfully, by looking at the difference in length of the female-specific 812 bp fragment and the homologous, longer fragment amplified for males. Gel electrophoresis results for a total of 81 Komodo dragons are reported for Ragunan zoo (Figure 6), Surabaya zoo (Figure 7), and Gembira loka zoo (Figure 8). First column of the Figures 6A-D, 7A-B, and 8 show a fragment size reference ladder (100 bp ladder). Tables 1, 2, and 3 show the animal identification number (ID) along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth there), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis.

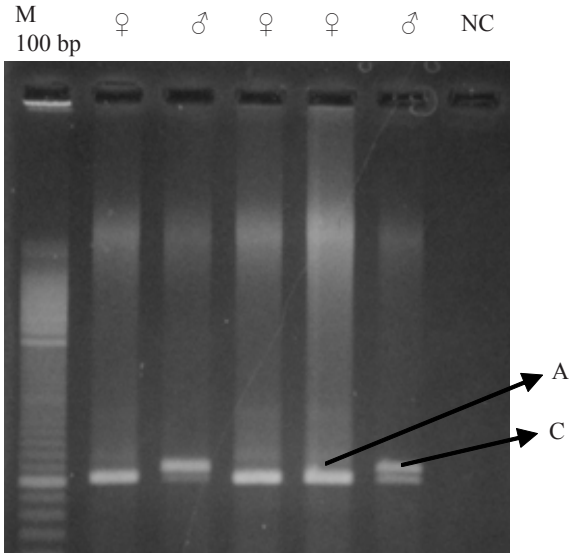

Figure 5. An example of the pattern of PCR amplification products sex-related DNA fragments. (M 100 bp = 100 bp DNA marker, ♀: Female, ♂: Male, NC: negative control).

Ragunan Zoo. Jakarta's zoo (Ragunan zoo) is situated in the suburb of Ragunan in the southern part of Jakarta and it has a captive breeding program for endangered species including Komodo dragons. In this study, the sex of 44 Komodo dragons kept in Ragunan zoo was determined (100%). A summary of sex determination results are reported in Table 1. The analysis identified 30 females and 14 males (Figure 6). The position of bands, for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

Surabaya Zoo. Surabaya Zoo is located in Indonesia's East Java province, where an artificial

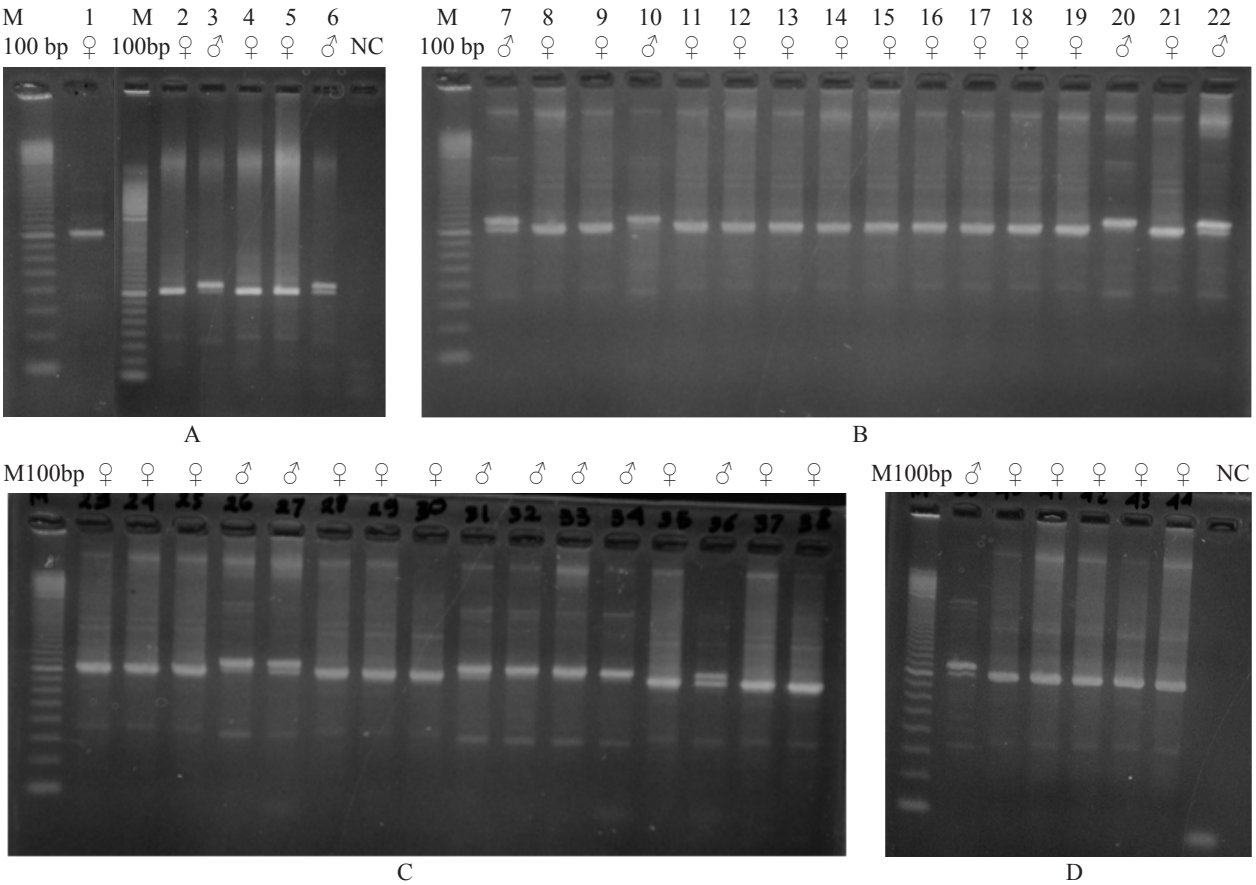

Figure 6A-D. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Ragunan zoo, in Jakarta. M 100 bp = 100 bp DNA marker, ♀: Female, ♂: Male, NC: negative control.

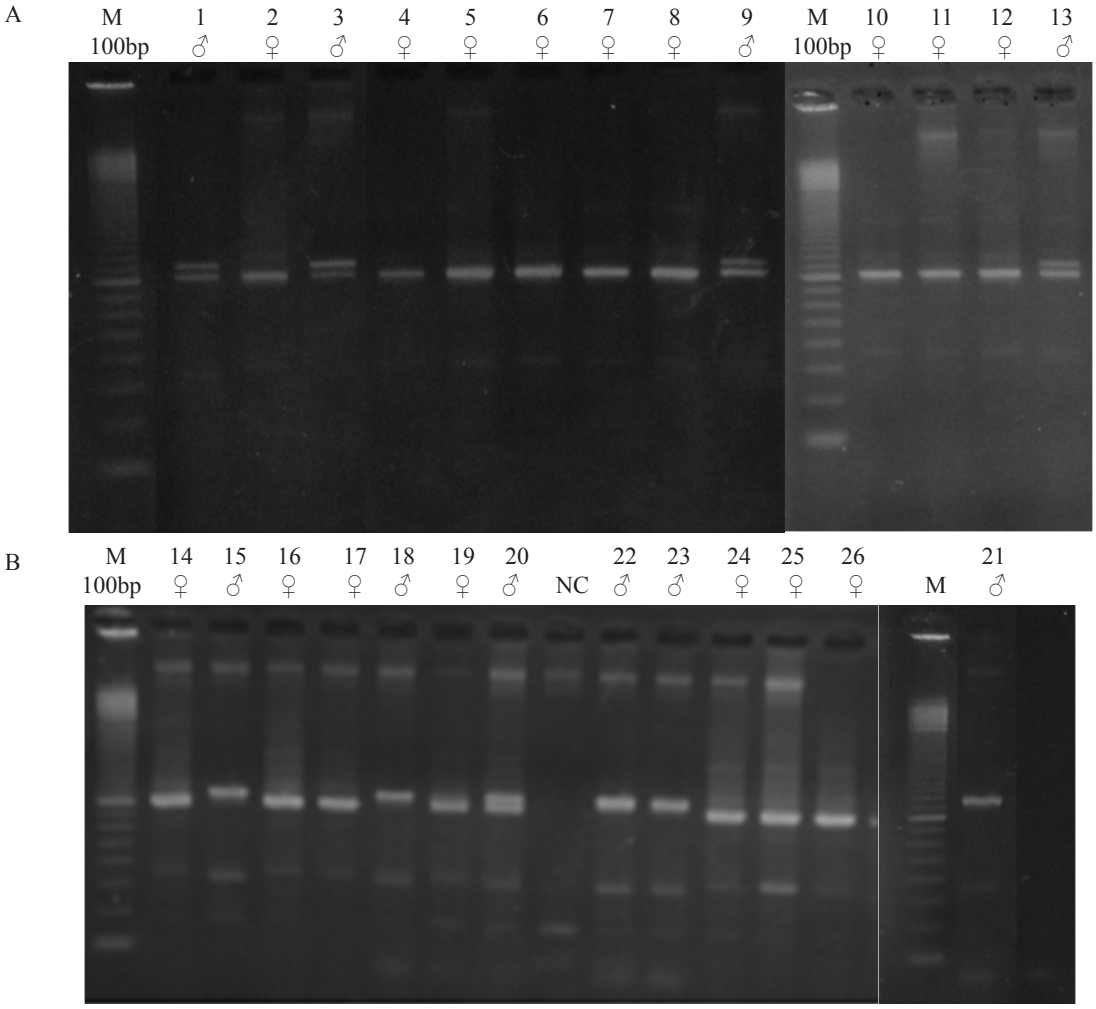

Figure 7A-B. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Surabaya zoo, in Surabaya. M 100 bp: 100 bp DNA marker, ♀: Female, ♂: Male, NC: negative control.

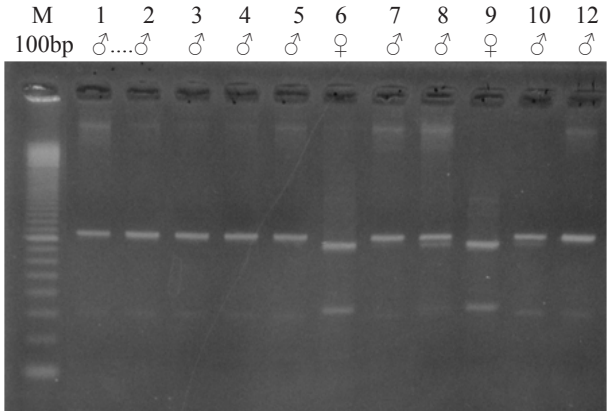

Figure 8. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Gembira Loka zoo, in Yogyakarta. M 100 bp: 100 bp DNA marker, ♀: Female, ♂: Male.

breeding program is underway for Komodo dragons. The sex of 26 komodo dragons kept in Surabaya zoo was determined (100%). Summary of sex determination results are reported in Table 2. Analysis identified 16 females and 10 males (Figure 7). The position of bands for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

Gembira Loka Zoo. The Gembira loka zoo has a large collection of dragons distributed in several cages. The sex of 11 komodo dragons kept in Gembira loka determined (100%). Summary of sex determination results are reported in Table 3. Our results identifed 2 females and 9 males (Figure 8). The position of bands for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

DISCUSSION

For animal marking, scientists have often relied upon external tags (e.g. ear and leg bands, dart tags, painted marks, scale clipping), which can also facilitate tracking of single individuals. Such tags are relatively cost-effective, but their external nature makes them susceptible to being lost, scratched,

rendered illegible upon recapture, and also subject to human error while being read (Gibbons & Andrews 2004). Branding, which has been used on such animals as elephants seals, often causes pain and has thus been viewed as inhumane (Galimbert & Sanvito 2000). PIT tags, by contrast, are placed under the skin and so are not as susceptible to damage or loss. They are read by computer scanners, avoiding human error; and they are safe for the animal as they rarely result in infection. Upon the death of the animal, tags can be removed and reused (Neubaum *et al*. 2005; Semmens *et al*. 2007).

The use of internal PIT tags began in the mid-1980s with scientists measuring fish movement, but has since been expanded for use in studying the movement of mammals, amphibians, reptiles, birds and invertebrates (Gibbon & Andrews 2004). PIT tags are also used by veterinarians for identifying household pets and for tracking livestock and zoo animals. Essentially, PIT tags act as a lifetime barcode for an individual animal, analogous to a social security number, and provided they can be scanned, they are as reliable as a fingerprint (Gibbon & Andrew 2004). In this study, a total of 81 Komodo dragons (from Ragunan zoo, Surabaya zoo, and Gembira loka zoo) were tagged as shown in Figure 2 using a micro-sized passive integrated transponder (PIT). Each transponder contains a 10 digit number which uniquely identifies the tagged animal (presented in Tables 1, 2, and 3), and which can be read using an appropriate scanner. After tagging, blood samples were taken individually for DNA sex determination.

Snout-vent Length (SVL) is a Standard Measurement of Body Length. Body length measurements were obtained from 81 Komodo dragons at Indonesian Zoos (Gembira loka, Surabaya, and Ragunan). According to Laver *et al*. (2012), Komodo dragons grow until they reach breeding age (about 7 years) after which the growth trajectories diverge, with females tending towards a smaller maximum size than males. Male Komodo dragons continue to grow until reach 160 cm in SVL size (a total length of 3 meters). On the other hand, females stop growing about 120 cm in SVL size (a total length of 2.2 to 2.5 meters). They also concluded that male Komodo dragons keep growing for competiting (fighting) with other males stop growing and divert energy for nesting purposes.

Sex determination in Komodo dragons has proved difficult under field conditions ["*for reptile biologists*"]. According to Morris *et al*. 1998, measurements of circulating testosterone and two-dimensional ultrasound imaging together appear useful in the noninvasive determination of gender in juvenile *V. komodoensis* at just over two years of age. Ultrasound procedures have been developed for the Komodo dragon to visualize the internal gonads to determine the sex of the animal. However, this procedure requires an anaesthesia, which can cause unnecessary stress for the animal.

DNA-based sex determination is ideal for sexing animals–especially those that are young and morphologically indistinguishable–since it only requires a small sample such as a drop of blood or a single hair, thereby minimizing animal trauma (Vali & Doosti 2011). Recently, the molecular genetics laboratory of zoology at the Research Center for Biology-LIPI was able to use DNA markers to determine the sex of Komodo dragons. DNA markers linked to the heterogametic sex chromosome was found to provide an easy diagnostic tool for identifying gender.

This study aimed at identifying the gender of captive Komodo dragons by amplifying hetero chromosome sequences of DNA obtained from blood samples collected in the three largest Zoos in Indonesia. In the ideal sex determination test, the final product is an agarose gel showing one band for the homogametic sex and two for the heterogametic sex. In Komodo dragons, females are heterogametic (they have one Z chromosome and one W chromosome), and DNA samples are therefore characterized by alleles of different size, detected in the gel as two distinct bands. Males are homogametic (they have two Z chromosomes) and have alleles of the same size, so that PCR products will run at the same speed during electrophoresis. The two alleles will overlap each other in the gel, and will be therefore represented by a single band. This is achieved through the use of primers that anneal to both sex chromosomes but amplify PCR products of different lengths. Komodo dragons are like birds, males are ZZ and females are ZW. As with the Y chromosome, the W chromosome is a minor chromosome with few functions beyond sex determination. When breeding, a female Komodo dragon can pass on either a Z or W chromosome to her offspring, while a male Komodo dragons can only pass on a Z chromosome.

Interestingly, Watts *et al*. (2006) presented evidence that the Komodo dragon can also produce parthenogenetically (virgin birth). Chapman *et al*. (2007) similarly reported facultative parthenogenesis in hammer-head sharks (*Sphyrna tiburo*). Parthenogenesis is a form of asexual reproduction in which growth and development of embryos occur without fertilization. In both

cases, the parthenogenetic events result in progeny homozygous for all loci, suggesting automictic parthenogenesis with terminal fusion and no recombination (Lenk *et al*. 2005). Watts *et al*. (2006) used genetic fingerprinting to identify parthenogenetic offspring produced by two female Komodo dragons (*Varanus komodoensis*) that had been kept at separate institutions and isolated from males; one of these females subsequently produced additional offspring sexually.

A female Komodo dragon kept alone will have spontaneous genome duplication of an egg. The female Komodo dragon, however, uses the ZW sex determination system, so the egg could either have a Z chromosome and duplication to be a ZZ male, or it could have a W chromosome and duplication to become an unviable WW embryo. In practice, therefore, this means that female Komodo dragons that revert to parthenogenesis will always generate ZZ males. Philip (2007) stated that a single female and her male progeny could start a population, which would have both lowered genetic variation and genetic load. Parthenogenesis in captive populations could also result in unexpectedly high inbreeding, loss of genetic variation and changes in founder contribution.

Ideally a sex-specific chromosome sequence discovered in the Komodo dragon would have utility for other monitor lizards. Unfortunately, the ancient lineage of reptiles has resulted in a lack of conservation of modes of sex determination. It is conjectured that reptilian sex determination has evolved many times. According to Halverson and Spelmann (2002) reptiles exhibit different modes of sex determination including both temperature-dependent determination and chromosomal sex determination. In regards to chromosomal determination some genera have XY sex chromosome, some genera have ZW sex chromosome, and some genera have no recognizable sex chromosome. It is indicated that some reptile families show all three variations. Therefore, it is unlikely that a DNA marker found in one species or genus will be sufficiently conserved to be broadly applicable to other reptiles.

The sex of Komodo dragons from Surabaya zoo, Ragunan zoo and Gembira loka zoo was evaluated in this study by using a protocol developed by our researchers. This was necessary because PCR reactions we attempted, using the settings and original conditions described in Halverson and Spelmann (2002), gave inconsistent results. Our researchers tested different protocols, varying the annealing temperatures, number of cycles, and concentrations of reagents, in order to identify the best protocol for sex determination using our sample set. This specially developed procedure resulted in the amplification of band A in females and band C in males (Figure 5). However, for females, band B was not always visible in this study and, sometimes, a similar, but lighter band was also amplified in males. The sexing results produced by Halverson and Spelmann (2002) showed two clear bands in females, i.e. band A and B, and one band in males. In this study, PCR always resulted in the amplification of the male-specific longer fragment (Figures 6, 7, 8) and the 812 bp fragment specific for females. Thus length of fragment was indicative of sex, rather than the number of bands.

In this study, DNA technology has proved to be a viable technique for sex determination of Komodo dragons, and useful for captive breeding, an important aspect of species conservation.

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